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Protectin DX Increases Alveolar Fluid Clearance In Rats With Lipopolysaccharide-Induced Acute Lung Injury

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ABSTRACT

Acute respiratory distress syndrome is a life-threatening critical syndrome resulting largely from the accumulation of and inability to clear pulmonary edema. Protectin DX, as an endogenously produced lipid mediator, is believed to exert anti-inflammatory and pro-resolution actions. Protectin DX (5ug/kg) was injected i.v. 8 h after LPS (14mg/kg) administration and alveolar fluid clearance was measured in live rats (n=8). In primary rat ATII epithelial cells, Protectin DX (3.605×10^{-3}mg/L) was added to the culture medium with LPS for 6 h. Protectin DX improved alveolar fluid clearance (9.65±1.60 *vs.* 15.85±1.49, $p<0.0001$) and decreased pulmonary edema and lung injury in LPS-induced lung injury in rats. Protectin DX markedly regulated alveolar fluid clearance with up-regulating the protein expression of sodium channel and Na,K-ATPase in vivo and in vitro. Protectin DX also increased the activity of Na,K-ATPase and up-regulated P-Akt via inhibition of Nedd4-2 in vivo. Besides, Protectin DX enhanced the subcellular distribution of sodium channel and Na,K-ATPase, specifically localized to the apical and basal membrane of the primary rat ATII cells. Furthermore, BOC-2, Rp-cAMP, and LY294002 blocked the increased alveolar fluid clearance response to Protectin DX. Protectin DX stimulates alveolar fluid clearance through a mechanism partly dependent on alveolar epithelial sodium channel and Na,K-ATPase activation via the ALX/PI3K/Nedd4-2 signaling pathway.

Key Words: Acute respiratory distress syndrome; Pulmonary edema; alveolar type II cell; Protectin

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a devastating clinical syndrome characterized by alveolar epithelial injury leading to non-cardiogenic pulmonary edema of flooding protein-rich in the fluid alveolar spaces[1,2]. Although our understanding of the pathophysiological changes associated with ARDS has improved now, there is still none effective management of this condition and mortality remains approximately 40%[2]. It is reported that alveolar fluid clearance is impaired in the majority of patients with ARDS[3]. Hence, timely and effective removal of excessive alveolar edema fluid is great importance for the better clinical outcomes[4].

Alveolar fluid clearance relys on active ion transport, which leads to anosmotic gradient that drives the movement of fluid from the alveolar space back into the interstitium and eventually to the blood circulation[5]. The mechanism of alveolar fluid clearance is the alveolar fluid removed by active Na^+ transport across the alveolar epithelium via an apical alveolar sodium channel[6] and through basolateral Na,K-ATPases[7]. Although therapies such as A_2B adenosine receptor[8], angiotensin[9], Triiodo-L-thyronine[10], Estradiol[11], have shown promising clinical effects in animal models, these have failed to translate positively in human studies[12]. We previously reported that an intravenous β-agonist (salbutamol) decreased extravascular lung water in ARDS patients[13,14]. However, we found that the side effects of salbutamol, such as tachycardia, arrhythmia, and lactic acidosis significantly, increased the 28-day mortality rate in a multicenter, randomized, controlled clinical trial[15]. Therefore, new therapeutic agents

need to be identified.

Protectins are novel lipid mediators in anti-inflammation and resolution[16]. Protectin DX is one of Protectins, an isomer of protectin D1[17], is believed to exert anti-inflammatory properties including inhibition of neutrophil activation and regulating inflammatory cytokines. It is produced by an apparent double lipoxygenase-mediated reaction in murine perritonitis exudates, in suspensions of human leukocytes, or by soybean 15- lipoxygenase incubated with DHA[17,18]. The mean concentration of Protectin DX in human blood was below 25pg/ml[19]. The Protectin DX level in C57BL/6 macrophages was about 60pg/10^6 cells[20], in skeletal muscle of mice was 30pg/g, in liver was 100pg/g[21]. The recent study demonstrated that Protectin DX could block neutrophil infiltration in murine peritonitis by 20-25% at a dose of 1ng/mouse[18]. It is reported that Protectin DX could alleviate insulin resistance by activating a myokine-liver glucoregulatory axis[22]. Our group previous data suggested that posttreatment with 15-epi-16-parafluorophenoxy lipoxin A4 could attenuate LPS -induced acute lung injury in mice[23]. However, there are no studies up to now have addressed the effect of Protectin DX on pulmonary edema.

The present study tested the hypothesis that administration of Protectin DX will increase alveolar fluid clearance in LPS–injured rat lungs. Our secondary hypothesis was that augmented alveolar fluid clearance would also be associated with ATII cells sodium channel, Na,K-ATPase, P-Akt and Nedd4-2 stimulation after treatment with Protectin DX. Finally, as the receptors and downstream signalling pathways of Protectin DX are under investigated, we

investigated the effect of ALX receptor inhibitor (BOC-2), cAMP inhibitor (Rp-cAMP), cGMP inhibitor (Rp-cGMP), PI3K inhibitor (LY294002), and PKA inhibitor (H89) on alveolar fluid clearance in ARDS to gain a better understanding of the mechanisms.

METERIALS AND METHODS

Materials

Protectin DX, LY294002 (PI3K inhibitor), and H89 (PKA inhibitor) were from Cayman Chemical Company (Ann Arbor, MI). LPS (Escherichia coli serotype055:B5) was purchased from Sigma (St. Louis, MO). Interleukin-1 (IL-1), Interleukin-10(IL-10), Tumor necrosis factor-α, myeloperoxidase and cAMP ELISA kits were from R&D Systems (Minneapolis, MN). BOC-2 (ALX inhibitor), Rp-cAMP (cAMP inhibitor), and Rp-cGMP (cGMP inhibitor) were obtained from Biomol-Enzo Life Sciences (Farmingdale, NY). Anti-Na,K-ATPase α1 and β1were purchased from Abcam (Cambridge, MA), anti- sodium channel α, β, γ were purchased from Biorbyt (Cambridge, Cambridgeshire). Anti- P-Akt and Total Akt (T-Akt) and Nedd4-2 were obtained from Cell Signaling Technology (Beverly, MA).

Animal preparation

Specific pathogen-free adult male Sprague-Dawley rats, weighing 250-300 g, obtained from Slac Laboratory Animal (Shanghai, China), were housed under controlled temperature and humidity in a day-night cycle, with free access to food and water with the Guide for the Care and Use of Laboratory Animals. The study was approved by the Animal Studies Ethics Committee of Wenzhou Medical University.

Rats were randomized into ten groups (n=8): Control group, LPS group, LPS+Alcohol group (Protectin DX's solvent, 50ul/kg), PDX group, LPS+PDX group, LPS+PDX+BOC-2 group, LPS+PDX+RP-cAMP group, LPS+PDX+ RP-cGMP group, LPS+PDX+H89 group and LPS+PDX+LY294002 group (PDX= Protectin DX). The LPS-induced lung injury model was produced by 14 mg/kg of LPS injected via caudal vein. In Protectin DX group, Control group, rats received Protectin DX (5ug/kg) or equivalent volume of saline via caudal vein. In LPS+PDX group, LPS+Alcohol group, rats received Protectin DX or alcohol (50ul/kg) via caudal vein 8 h after LPS exposure. In LPS+PDX+BOC-2 group, LPS+PDX+RP-cAMP group, LPS+PDX+RP-cGMP group, LPS+PDX+H89 group and LPS+PDX+LY294002 group, rats received Protectin DX with BOC-2 (600ng/kg), or RP-cAMP (5mg/kg), or RP-cGMP (5.5 mg/kg), or H89 (10 mg/kg), or LY294002 (3mg/kg) via caudal vein 8 h after LPS exposure. Before a tracheotomy tube was placed, rats were anesthetized with an i.p. injection of 5% chloral hydrate (7 ml/kg). Subsequently, Rats were sacrificed after sustained 60 minutes of mechanical ventilation was provided to them, and lungs were harvested.

Pathological studies

The right lower lung lobes were harvested and fixed in 4% paraformaldehyde for 24 h, then embedded in paraffin and stained with hematoxylin and eosin (H&E) for light microscope analysis. A semi-quantitative scoring system was adopted to evaluate the lung injury including alveolar congestion, alveolar hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation and

inflammatory cell infiltration. The grading scale of pathologic findings was used in a light microscope as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). The results were graded from 0 to 4 for each item, as described previously[27,28]. The four variables were summed to represent the lung injury score (total score: 0-16).

Part of the right lung was homogenized from individual rats and centrifuged, and the tissue level of myeloperoxidase, TNF-α, IL-1, IL-10 in the resulting supernatants was respectively determined using myeloperoxidase, TNF-α, IL-1 and IL-10 ELISA kit.

Transmission electron microscopy

Blocks were rinsed overnight in 0.1 M phosphate buffer (350 mOsm, pH 7.4) and postfixed for two hours in osmium tetroxide (1% osmium tetroxide in 0.125 sodium cacodylate buffer; 400 mOsm, pH 7.4). The samples were then passed through stepwise dehydration in increasing concentrations of ethanol (50-100 percent), rinsed with propylene oxide and embedded in Araldite. Blocks were then cut into ultra thin sections (50-70 nm) and contrast stained with saturated uranyl acetate and bismuth subnitrate. Sections were examined at an accelerating voltage of 60 kV using a Zeiss EM 10C transmission electron microscope. Micrographs of a carbon grating replica were taken for calibration.

Measurement of alveolar fluid clearance in live rats

Alveolar fluid clearance was measured in living rats as previously described[11,29,30] with some modifications. Clearance is expressed as a percentage of total instilled volume cleared after 60

161 min. Alveolar fluid clearance was determined by Evans blue-tagged albumin concentration
162 changes, which has been clearly characterized by our laboratory[31].
163 For preparation of the alveolar instillate, a 5% albumin instillate solution was prepared by
164 dissolving 50 mg/ml BSA in modified lactated Ringer's solution: 137 mM NaCl, 4.67 mM
165 KCl, 1.82 mM $CaCl_2$*$2H_2O$, 1.25 mM $MgSO_4$*$7H_2O$, 5.55 mM dextrose, and 12 mM HEPES.
166 The pH was adjusted to 7.4 at 37˚C. The albumin solution was labeled with 0.15 mg/ml Evans
167 blue. In brief, after anesthesia with 5% chloral hydrate (7ml/kg), a polyethylene endotracheal
168 tube was inserted through a tracheotomy. Rats were ventilated with a constant volume
169 ventilator (model HX-300 Animal ventilators; Taimeng Company of Chengdu, China) with an
170 inspired oxygen fraction of 100%, a respiratory rate of 45-50 breaths/min and 4.5±0.2 ml tidal
171 volumes, positive end expiratory pressure was kept at 2-3 cm H_2O during the baseline period.
172 After tracheotomy, the rats were allowed to stabilize for 10 min. The animals were then
173 placed in the left lateral decubitus position, and instillation tubing (16G Epidural catheter)
174 was gently passed through the tracheotomy tube into the left lung. A total of 1.5ml (5ml/kg) of
175 the instillate solution was instilled at a rate of 0.08 ml/min using a syringe pump. After
176 instillation was complete, 0.2 ml air was injected to clear the instillation catheter of liquid.
177 The instillate remaining in the syringe was collected as the initial sample. After instillation,
178 the catheter was left in place for a duration of 60 min. The final alveolar sample was collected
179 via the instillation catheter. The concentrations of Evans blue-labeled albumin in the instilled
180 and aspirated solutions were measured by a spectrophotometer at a wavelength of 621 nm.

Alveolar fluid clearance was calculated using the following equation: alveolar fluid clearance = $(1-C_0/C_1)$, where C_0 is the protein concentration of the instillate before instillation, and C_1 is the protein concentration of the sample obtained at the end of 60 min of mechanical ventilation.

Primary rats ATII cells isolation, culture, and treatment

Primary rats ATII cells were isolated from Sprague-Dawley rats (250-300g) by elastase digestion of lung tissue and then differentially adhered on IgG-coated plates as described by Dobbs et al[32].The purity of ATII cells was assessed by modified Papanicolaou stain based on the presence of dark blue inclusions. Cell viability was assessed by trypan blue exclusion (>95%). ATII cells were seeded onto plastic culture dishes at $1\times10^6/cm^2$ and cultured in a 5% CO_2, 95% air atmosphere in dulbecco's modified eagle medium containing 10% fetal bovine serum, 2 Mm L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin after isolation. For all experiments, cells were subcultured into six-well plates and maintained until subconfluence (80%), and cells were serum deprived for 24 h before the addition of LPS (1ug/ml) in the presence or absence of Protectin DX (3.605×10^{-3}mg/L).

Western blotting for sodium channel, Na, K-ATPase, P-Akt, Nedd4-2

Proteins were obtained with RIPA lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100,1% sodium deoxycholate, 0.1% sodium dodecyl sulfonate, sodium orthovanadate, sodium fluoride, ethylene diamine tetraacetic acid, leupeptin) and phenylmethanesulfonyl fluoride. Samples were ultrasonicated 3 times, for 5s, and then spun at

12,000×g/min for 30min. Protein concentration of the supernatants were determined by a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). Proteins were separated by 10% sodium dodecyl sulfonate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat dried milk in TBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies: sodium channel α, β, γ (1:500, 1:700, 1:800), Na,K-ATPase α1, β1 (1:500,1:1000), and β-actin (internal control, 1:500), Akt (1: 1000), P-Akt (1: 2000) and Nedd4-2 (1:1000) overnight at 4˚C, and then reacted with HRP-conjugated secondary Ab (1:000; Santa Cruz Company) at room temperature for 1.5 h. The protein bands were detected by electrochemiluminescence (ECL) and visualized by UVP Gel imaging system (Upland, CA). The band intensity was analyzed by AlphaEaseFC (version 4.0).

Confocal imaging

ATII cells were respectively treated with saline, LPS (1ug/ml), LPS+Alcohol (the same volume of Protectin DX), LPS+PDX (3.605×10^{-3}mg/L), and Protectin DX for 6h before fixing in 4% paraformaldehyde and blocked with PBS containing 10%donkey serum for 30 min. The cells were then incubated in a 1:50 dilution of monoclonal mouse anti-Na,K-ATPase α1, and goat anti- sodium channel α at 4°C for48 h, followed by Alexa Fluor donkey anti-goat and donkey anti-mouse IgG incubation (1:100 and 1:300; Jackson) at room temperature for 2 h. Cell images were acquired with confocal laser-scanning microscope (Leica) and analyzed by Image Pro plus 6.3 software (Media Cybernetics, Crofton, MA).

Measurement of Na,K-ATPase activity in rat lung tissues

The hydrolytic activity of Na,K-ATPase was measured as ouabain-sensitive ATP hydrolysis under maximal velocity conditions by measuring the release of inorganic phosphate from ATP, as previously described[33]. In brief, the rat lung tissues were digested, subjected to centrifugal sedimentation, lysed, and homogenized. The minimal ATP enzyme test kit (Jian cheng Company, Nanjing, China) was used to assay Na,K-ATPase activity following manufacturer's instructions.

Measurement of cAMP concentration

Lung samples were treated with isobutyryl methylxanthine(Sigma) to inhibit phosphodiesterases, homogenized in ice-cold1 M TCA, and then centrifuged at 2,500 *g* to precipitate particulate material. The cAMP content in the supernatant was measured via ELISA according to the manufacturer's instructions (R&D Systems).

Blinding Method

The present study adopted randomized, blinded methods. The randomization list of animals was computer-generated by the statistician using SAS/STAT software. Blinding was accomplished by separation of function: a treatment administrator was responsible for dispensation and administration of animal models. Others specialized in extraction of lung tissue for Pathological studies, Transmission electron microscopy study, alveolar fluid clearance measurement and Western blotting et al.

Statistical analysis

Data are represented as mean ± SD. There were not any missing, lost, or excluded data. Based on previous experience, no a priori power calculation was conducted, all data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All tests were two-sided, significance was determined at the $p<0.05$ level. Statistical analyses were performed using Prism 6.0 software (GraphPad Software, San Diego, CA).

RESULTS

Protectin DX protected lung tissues from LPS-induced acute lung injury *in vivo*

First, we evaluated the effect of Protectin DX (5ug/kg) on LPS (14mg/kg) -induced acute lung injury. The control group revealed normal pulmonary histology (Fig.1A,B). Compared with the control group, lung tissues in the LPS group were markedly damaged with interstitial edema, hemorrhage, and inflammatory cells infiltration as evidenced by an increase in lung injury score (Fig.1C). All the morphologic changes were less pronounced in the LPS+PDX group. Protectin DX significantly reduced LPS-induced pathologic changes by the evidence of a decrease in lung injury score. There was no significant difference between the control and Protectin DX groups (Fig. 1C).

In addition, the concentration of myeloperoxidase, TNF-α, IL-1 and IL-10 in the lung tissues homogenate were significantly increased in the LPS group compared with the control group, but reduced in the Protectin DX treatment group compared with the LPS group (Fig.1D,E,F,G).

Finally, we further tested Protectin DX actions on the ultrastructure of lung tissues. The lamellar bodies, which is the most significant diagnostic characteristic of ATII, were sever vacuolated in the LPS group compared with the control group, however, lamellar bodies recovered in the Protectin DX treatment group (Fig.2A). As shown in Fig.2B, the control group revealed normal air-blood barrier. In contrast, air-blood barrier in the LPS group was damaged, broken epithelial bridges and capillary wall appeared in tissues. In treatment group, Protectin DX significantly improved the air-blood barrier induced by LPS.

Protectin DX upregulated alveolar fluid clearance in LPS-induced acute lung injury *in vivo*

Next, we examined the effect of Protectin DX (5ug/kg) on alveolar fluid clearance in LPS (14mg/kg) -induced acute lung injury in vivo. In all concentration of Protectin DX (1.5, 2.5, 5 and 7.5μg/kg), 1.5ug/kg Protectin DX could not improve the alveolar fluid clearance reduced by LPS. 2.5μg/kg Protectin DX can improve the alveolar fluid clearance reduced by LPS, and reached the maximal effect at 5μg/kg, the effect of Protectin DX was similar between 5μg/kg and 7.5μg/kg. Therefore, Protectin DX at a concentration of 5ug/kg or vehicle alone was injected through caudal vein 8 h after LPS (14mg/kg) administration, and alveolar fluid clearance was determined after 60 min. Alveolar fluid clearance in the LPS group was as expected, reduced compared with the control group (9.65±1.60 *vs.* 19.23±1.20), whereas Protectin DX increased alveolar fluid clearance after LPS-induced acute lung injury (15.85±1.49 *vs.* 9.65±1.60). However, there was no significant difference between the control

and Protectin DX groups (Fig.2C).

Protectin DX enhanced sodium channel, Na,K-ATPase in LPS-induced acute lung injury *in vivo*

The protein expression of sodium channel α,γ subunits and Na,K-ATPase α1, β1 subunits, but not β subunit, were increased in the LPS+PDX group compared with the LPS group (14mg/kg) in rat lung tissue homogenates (Fig.3A-E). In addition, LPS markedly reduced the Na,K-ATPase activity compared with the control group, whereas Protectin DX (5ug/kg) heightened Na,K-ATPase activity 8 h after LPS-induced acute lung injury (Fig. 3F).

Protectin DX promoted alveolar fluid clearance through activating the ALX/cAMP/PI3Kpathway *in vivo*

To further investigate ALX/cAMP/PI3K-dependent actions of Protectin DX (5ug/kg) in vivo, firstly, we measured cAMP concentration in lung tissues. We found cAMP concentration was lessened in the LPS group (14mg/kg) compared with the control group, and Protectin DX treatment increased cAMP concentration in LPS treated lungs (Fig.4A). In addition, cAMP concentration were reduced in the LPS+PDX+BOC-2 group and LPS+PDX+LY294002 group compared with the LPS+PDX group (Fig.4B). Secondly, the protein expression of Ser^{473}-phos-phorylated Akt in rat lung tissue homogenates was measured by Western blotting. The protein level of phosphorylated Akt in the LPS group was markedly decreased compared with control group and observably increased in the LPS+PDX group compared with the LPS group. BOC-2 (600ng/kg) and LY294002 (3mg/kg) markedly suppressed the Protectin

DX-induced increased in the protein level of P-Akt (Fig.4C).

Nedd4-2, an E3 ubiquitin-protein ligase, is critical in the negative control of Na^+ transport. The protein level of Nedd4-2 in LPS group was significantly increased compared with the control group and significantly decreased in the Protectin DX treatment group compared with the LPS group (Fig.4D). Finally, we co-administered Protectin DX (5ug/kg) and BOC-2 (600ng/kg), Rp-cAMP (5mg/kg), Rp-cGMP (5.5mg/kg), LY294002 (3mg/kg) or H89 (10 mg/kg) to rats through caudal vein 8 h after LPS (14mg/kg) administration, and alveolar fluid clearance was determined after 60 min. Alveolar fluid clearance in the LPS+PDX+BOC-2 group (11.94±1.56), LPS+PDX+Rp-cAMP group (11.90 ± 1.36) and LPS+PDX+LY294002 group (10.81±1.49) was reduced compared with LPS+PDX group (15.85±1.49), whereas there was no significant changes in the LPS+PDX+Rp-cGMP and LPS+PDX+H89 groups (Fig.4E). The beneficial effect of Protectin DX on pulmonary histology was abrogated by treatment with BOC-2, Rp-cAMP and LY294002 (Supplementary Figure).

Dose and time dependency Protectin DX regulated Na,K-ATPase α1 expression in primary ATII cells

Different concentrations of Protectin DX including 3.605×10^{-4}, 3.605×10^{-3}, 18.025×10^{-3}, 3.605×10^{-1} mg/L was incubated with primary ATII cells. As shown in Fig.5A, the Na,K-ATPase α1 subunit expression was increased dose-dependently with a concentration of 3.605×10^{-3}mg/L producing a maximal effect. In subsequent experiments, the sodium channel and Na,K-ATPase expression in ATII cells was assessed using 3.605×10^{-3}mg/L Protectin DX.

The dynamic expression of Na,K-ATPase α1 subunit in primary II cells was significantly increased at 6h (Fig.5B).

Protectin DX increased the expression of sodium channel, Na,K-ATPase in primary rat ATII cells

In vitro, rat primary ATII alveolar epithelial cells were incubated with Protectin DX (3.605×10^{-3}mg/L) in the presence or absence of LPS (1ug/ml) for 6 h at 37°C. Protectin DX increased sodium channel α (Fig.6A) and Na,K-ATPase α1 (Fig.6B) by confocal laser-scanning microscopy. The protein expression of sodium channel α , γ subunits and Na,K-ATPase α1, β1 subunit, but not sodium channel β subunit, were increased in the LPS+PDX group compared with the LPS group (Fig. 6C, D).

DISCUSSION

In the present study, we have provided evidence for the pro-resolution actions of Protectin DX in acute lung injury. Treatment with Protectin DX improved alveolar fluid clearance and decreased pulmonary edema and lung injury in LPS-induced acute lung injury in rats. Protectin DX markedly regulated alveolar fluid clearance via up-regulating the protein expression of sodium channel and Na,K-ATPase in vivo and in vitro. Protectin DX also increased the activity of Na,K-ATPase in vivo. Besides, Protectin DX enhanced the subcellular distribution of sodium channel and Na,K-ATPase, specifically localized to the apical and basal membrane of the primary rat ATII cells. Furthermore, BOC-2, Rp-cAMP,

and LY294002 blocked the increased alveolar fluid clearance response to Protectin DX. The results above indicated that Protectin DX increased the expression of sodium channel and Na,K-ATPase to promote alveolar fluid clearance via the ALX/cAMP/Nedd4-2 signalingpathway.

Acute lung injury is a critical illness syndrome characterized by an increased permeability of the alveolar-capillary barrier resulting in impairment of alveolar fluid clearance[27]. So far, no specific therapy is currently available to modulate this inflammatory response[28]. It is widely accepted that resolution of alveolar edema is the crucial step to patient survival[4]. Our data clearly demonstrate that Protectin DX significantly improved the air-blood barrier and lamellar body structure challenged by LPS, especially enhanced the rate of alveolar fluid clearance 8 h after LPS challenge, and no effect on alveolar fluid clearance in healthy rat lungs, indicating that Protectin DX plays an important role in the resolution of inflammation.

It is well known that active Na^+ transport is the dominant ion transport mechanism involved in alveolar liquid clearance[5]. The cellular and molecular mechanisms responsible for the vectorial transport of Na^+ from the alveoli to the interstitium have been reasonably well defined[29]. sodium channel is the first constituent of the Na^+ transport system, it is expressed in both alveolar type II and type I cells[30]. Unable to clear alveolar edema fluid, sodium channel α gene knockout mice died within 40 h after birth[31]. Previously, we found that SPMs, such as lipoxin, resovin could improve the alveolar fluid clearance via regulating sodium channel, cystic fibrosis transmembrane conductance regulator (CFTR) or aquaporin. In our study,

Protectin DX not only enhanced lung tissues homogenate sodium channel α and γ subunits protein expression in LPS-induced acute lung injury, but also increased sodium channel α and γ subunits protein expression in primary ATII cells stimulated with LPS. Meanwhile, confocal laser-scanning microscopy results of primary ATII cells draw identical outcomes. Consistent with our findings, similar results have shown that up-regulation of sodium channel increased pulmonary edema fluid reabsorption and reduced sodium channel expression delayed reabsorption of fluid during pulmonary edema after thiourea-induced lung injury[32]. These findings, therefore, suggest that Protectin DX promotes alveolar fluid clearance through up-regulation the expression of sodium channel.

Na^+ enters the cell by the amiloride-sensitive sodium channel or by other cationic channels located at the apical surface, and is extruded by Na,K-ATPase located at the basolateral surface. It is reported that inhibition or loss of Na,K-ATPase could markedly decrease solute and fluid transport in alveoli[7]. Previous studies showed that up-regulation of sodium channel and Na,K-ATPase increased active Na^+ transport, leading to increased ability of the lungs to clear edema[27,33]. Impairment of Na,K-ATPase function appears to be a hallmark during lung injury even in a preclinical stage[27,34]. In our study, we demonstrated that Protectin DX not only increased Na,K-ATPase α1,β1 expression in rat lung tissues and primary ATII cells after LPS challenge by Western blotting and confocal laser-scanning microscopy measurement, but also up-regulation of Na,K-ATPase activity in vivo. Together, the lung tissues and cell culture data indicate that Protectin DX promotes alveolar fluid clearance through both of the essential

mechanisms of sodium channel and Na,K-ATPase.

Specialized pro-resolving mediators (SPMs) derived from ω-3 polyunsaturated fatty acids orchestrate resolution in diverse settings of acute inflammation[35]. As one of SPMs family, the side effect of Protectin DX in low-dose was little, but the high-dose of Protectin DX may induce calcium ions inflex. So far the receptors and downstream signaling pathways of Protectin DX are under investigated. It has been generally believed that SPMs exert their actions by interacting with G-protein-coupled receptor (GPCR) with high affinity and stereospecificity[36,37]. Previously, two G protein-coupled receptors (GPCRs) of Resolvin D1 were identified, and validated using a GPCR/arrestin-coupled system, namely, OrphanGPR32 and ALX (the lipoxin A4 receptor, formyl-peptide receptor type 2 [FPR2], also called ALX)[38]. It was the first receptor cloned and identified as a GPCR for lipoxin and resolvin with demonstrated cell-type-specific signalling pathways[39,40]. In our study, Protectin DX increased alveolar fluid clearance, but the beneficial effects were abrogated by ALX antagonist (BOC-2) in vivo, suggesting that the Protectin DX response is ALX dependent.

cAMP and cGMP are important second messengers by which cells transduce extracellular signals into intracellular responses pathways. Extracellular signals interact with GPCRs to activate the adenylate cyclase and increase the intracellular cAMP levels. A previous study showed that LPS-induced immune response leaded to a decrease of intracellular cAMP. Another study showed that stimulation with cAMP not only increased Na^+ transport within 5 min, but also promoted Na,K-ATPase recruitment to the plasma membrane[41,42]. Extracellular

signals also interact with GPCR to activate theguanylyl cyclases and increase the intracellular cGMP levels. In this context, there is evidence for substantial compartmentalization of two signaling pathways on regulating alveolar fluid clearance, cAMP and cGMP. In accordance with previously, the intracellular cAMP level was decreased after LPS stimulation, and Protectin DX abrogated the decrease observed in the LPS group in vivo. As further proof, Rp-cAMP and Rp-cGMP were used in vivo, of interesting, we found that the Rp-cAMP, not Rp-cGMP, reduced alveolar fluid clearance in LPS-induced acute lung injury, indicating that Protectin DX promoted alveolar fluid clearance by activating cAMP via ALX, but not cGMP.

It is well-known that PI3K signals are implicated in regulating sodium channel trafficking and activity[43]. The PI3K has been identified for regulation of sodium channel-mediated alveolar fluid clearance by insulin[44]. Against this background, our purpose was to evaluate whether the role of Protectin DX on lung is ALX/cAMP/PI3K dependent. Our studies illustrated that the intracellular cAMP level was decreased after LPS stimulation, and Protectin DX abrogated the decrease observed in the LPS group in vivo. Furthermore, we found that Protectin DX -induced increase in the levels of cAMP blocked with BOC-2and LY294002. Moreover, BOC-2 and LY294002 blocked the increased alveolar fluid clearance in Protectin DX treatment group. These results, taken together, manifesting that the Protectin DX response is ALX/cAMP/PI3K dependent.

It is well accepted that Akt is one of the signaling cascade downstream of PI3K, which is believed to be the central mediator of signaling with profound effects on several physiological

events[45,46]. Our studies revealed that P-Akt (ser[473]) was decreased after LPS stimulation and Protectin DX reversed the decreased of P-Akt (ser[473]), but the beneficial effects were abrogated by BOC-2 and LY 294002. The implication for our work is that Protectin DX promoted alveolar fluid clearance by PI3K/Akt signalling pathway.

Nedd4-2, which has been shown to negatively regulate sodium channel expression in vitro and in vivo[47,48]. Recent studies using Nedd4-2-deficient mice clearly demonstrated that Nedd4-2, which is co-expressed with sodium channel in lung epithelial cells transporting Na^+, plays a crucial role in the regulation of sodium channel activity in the lung[49]. Another study of Fisher rat thyroid cell proved the regulation of α-, β- and γ-sodium channel heterologously expressed via PI3K/Akt pathway by suppression of Nedd4-2[54].Consistently, we also found Protectin DX inhibited the increase of Nedd4-2 protein expression induced by LPS, the beneficial effect of Protectin DX on reducing Nedd4-2 protein expression was abolished by BOC-2 and LY294002 in vivo.

Our study demonstrates that Protectin DX alleviated pulmonary edema, enhanced alveolar fluid clearance, and attenuated lung injury partially through stimulation of sodium channel and Na,K-ATPase via activation of the ALX/PI3K/Nedd4-2 pathway in LPS-induced acute lung injury without affecting normal lung(Fig.7). Thus, treatment with Protectin DX in critically ill patients with acute lung injury has the potential to augment lung edema clearance. Our findings reveal a novel mechanism for pulmonary edema fluid reabsorption and Protectin DX may provide a new therapy for the resolution of ARDS.

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Conflict of Interest: Conflict of Interest: No conflict of interest exists for any of the above named authors contributing to this work.

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595 **FIGURE LEGENDS**

596 **Figure 1 - Protectin DX protected lung tissues in LPS-induced acute lung injury**.

597 Protectin DX (5ug/kg) was administered to Sprague-Dawley rats 8 h after LPS (14mg/kg)
598 stimulation through caudal vein, ventilating for 60 min, and the effect of Protectin DX was
599 assessed (A,B) by histology in hematoxylin and eosin -stained sections(original magnification
600 ×100, ×400). Lung injury scores (C) were recorded from0 (no damage) to 16 (maximum
601 damage) according to the criteria described in Materials and Methods. Lung tissues
602 myeloperoxidase (D), TNF-α (E), IL-1 (F), IL-10 (G) expression were measured by Elisa to
603 quantitatively define the resolution of infiltrated cells.

604 Data are presented as mean ± SD. n = 8. PDX= Protectin DX. MPO=myeloperoxidase.
605 Alcohol is Protectin DX's resolvent. $^{**}p<0.01$ versus control group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ versus
606 LPS group; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ versus LPS+Alcohol group.

607 **Figure 2 - Effect of Protectin DX on the ultrastructure of lung tissues and alveolar fluid**
608 **clearance in LPS-induced acute lung injury *in vivo***

609 Protectin DX (5ug/kg) was administered to Sprague-Dawley rats 8 h after LPS (14mg/kg)
610 stimulation through caudal vein, ventilating for 60 min, and the effect of Protectin DX was
611 assessed by electronmicroscope photomicrographs of lung tissue from rats. The lamellar
612 bodies in the LPS group were sever vacuolated compared with the control group, however
613 lamellar bodies recovered in the Protectin DX treatment group (A). The control group
614 revealed normal air-blood barrier. In contrast, air-blood barrier in the LPS group was damaged,

615 broken epithelial bridges and capillary wall appeared in tissues. In treatment group, Protectin
616 DX significantly improved the air-blood barrier induced by LPS (B). lb, lamellar body; bc,
617 blood capillary; ac, air capillary; ep, epithelial bridge; en, endothelial cell; ecm, extracellular
618 matrix of the capillary wall; cn, cell nucleus; er, erythrocyte.

619 After intratracheal instillation of 5% albumin solution containing Evans blue-labeled albumin
620 (5ml/kg) through a tracheostomy to the left lung, alveolar fluid clearance was measured over
621 60 min in ventilated animals. Data are presented as mean ± SD. n = 8. PDX= Protectin DX.
622 Alcohol is Protectin DX's resolvent. ** $p<0.01$ versus control group; $^{\dagger\dagger}p<0.01$ versus LPS
623 group; $^{\ddagger\ddagger}p<0.01$ versus LPS+Alcohol group.

624 **Figure 3- Protectin DX enhanced sodium channel, Na,K-ATPase expression and**
625 **Na,K-ATPase activity in LPS-induced acute lung injury *in vivo.***

626 Protectin DX (5ug/kg) was administered to Sprague-Dawley rats 8 h after LPS (14mg/kg)
627 stimulation through caudal vein, ventilating for 60 min, and the right lung tissue was
628 harvested to measure the protein expression of sodium channel α, β and γ subunits (A,B,C)
629 and Na,K-ATPase α1 and β1 subunits (D,E) by Western blotting. In addition, the
630 Na,K-ATPase activity in lung tissue homogenate was detected by kits (F). Data are presented
631 as mean ± SD. n =8. PDX= Protectin DX. Alcohol is Protectin DX's resolvent. *$p<0.05$,
632 **$p<0.01$ versus control group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ versus LPS group; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$
633 versus LPS+Alcohol group.

634 **Figure 4–Protectin DX improved alveolar fluid clearance was partly dependent on ALX,**

cAMP and PI3K pathway *in vivo*.

Protectin DX (5ug/kg) and BOC-2 (ALX receptor inhibitor, 600ng/kg), LY294002 (PI3K inhibitor, 3mg/kg), Rp-cAMP (5mg/kg), Rp-cGMP (5.5mg/kg) or H89 (10mg/kg) were co-administered to Sprague-Dawley rats 8 h after LPS (14mg/kg) stimulation through caudal vein, and intratracheal instillation of 5% albumin solution containing Evans Blue-labeled albumin (5ml/kg) through a tracheostomy to the left lung to measure alveolar fluid clearance (E); and the right lung tissue was harvested to measure the cAMP concentration by ELISA kits (A,B), the protein expression of phosphorylation of Akt (C) and Nedd4-2 (D) by Western blotting. Data are presented as mean ± SD. n=8. PDX= Protectin DX. Alcohol is Protectin DX's resolvent. $^{**}p<0.01$ versus control group; $^{\dagger\dagger}p<0.01$ versus LPS group; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ versus LPS+PDX group.

Figure 5-Dose and time dependency of Protectin DX regulated Na,K-ATPase expression in primary ATII cells.

The dose- and temporal-dependent changes of Na,K-ATPase protein expression in primary ATII cells stimulated with LPS (1ug/ml) were determined by Western blotting. Cells were incubated with different concentrations of Protectin DX for 6 h including 3.605×10^{-4}, 3.605×10^{-3}, 18.025×10^{-3} and 3.605×10^{-1} mg/L to measure the Na,K-ATPase α1 subunit protein expression (A). Moreover, ATII cells were incubated with LPS (1ug/ml) for 1, 2, 4, and 6 h to detect the expression of Na,K-ATPaseα1 subunit protein (B). Data are presented as mean ± SD. n =8. PDX= Protectin DX. $^{*}p<0.05$, $^{**}p<0.01$ versus control group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$

versus LPS group.

Figure 6- Protectin DX promoted sodium channel expression in primary rat ATII cells stimulated with LPS.

Rat primary ATII cells were treated with Protectin DX (3.605×10^{-3}mg/L) in the presence or absence of LPS (1ug/ml) for 6 h. After incubation, cells were harvested and sonicated. Sodium channel α (A) and Na,K-ATPase α1 (B) subunits protein expression in the cell lysates were detected by confocal laser-scanning microscopy using a specific Ab against (original magnification ×400). In addition, sodium channel α, β, and γ subunits (C) and Na,K-ATPase α1 and β1 subunits (D) protein expression in the cell lysates were detected by Western blotting. Data are presented as mean± SD. n = 8. PDX= Protectin DX. Alcohol is Protectin DX's resolvent. $^{**}p<0.01$ versus control group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ versus LPS group; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ versus LPS+Alcohol group.

Figure 7- Protectin DX protected LPS-induced acute lung injury *in vivo* and *in vitro*.

Supplementary figure legends YES

Figure S1. Different inhibitors on histological signs of lung injury

Figure 1

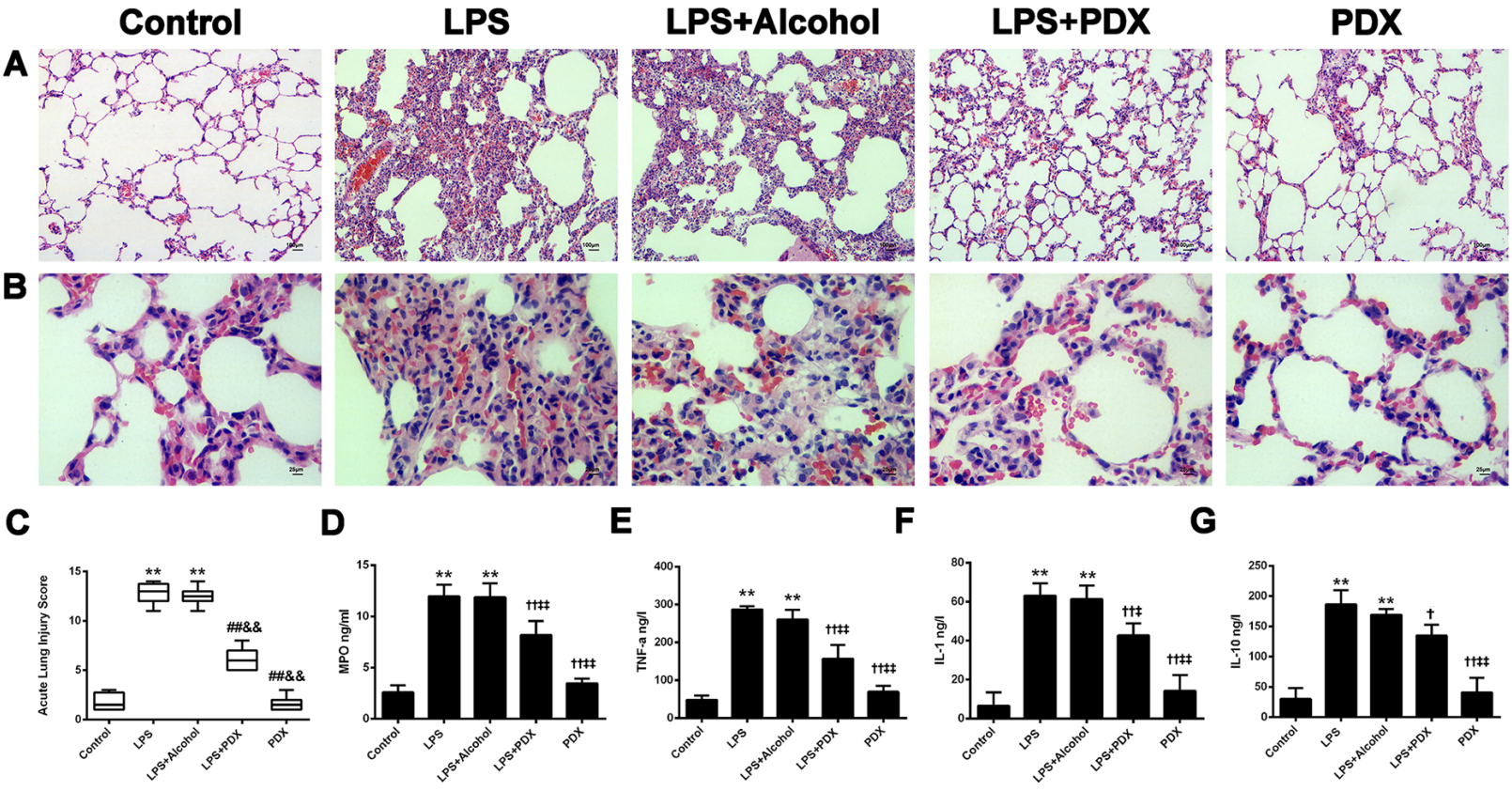

Control
LPS
LPS+Alcohol
LPS+PDX
PDX
A
B
C
D
E
F
G
Acute Lung Injury Score
MPO ng/ml
TNF-a ng/l
IL-1 ng/l
IL-10 ng/l
Control
LPS
LPS+Alcohol
LPS+PDX
PDX
**
##&&
†††‡

Figure 2

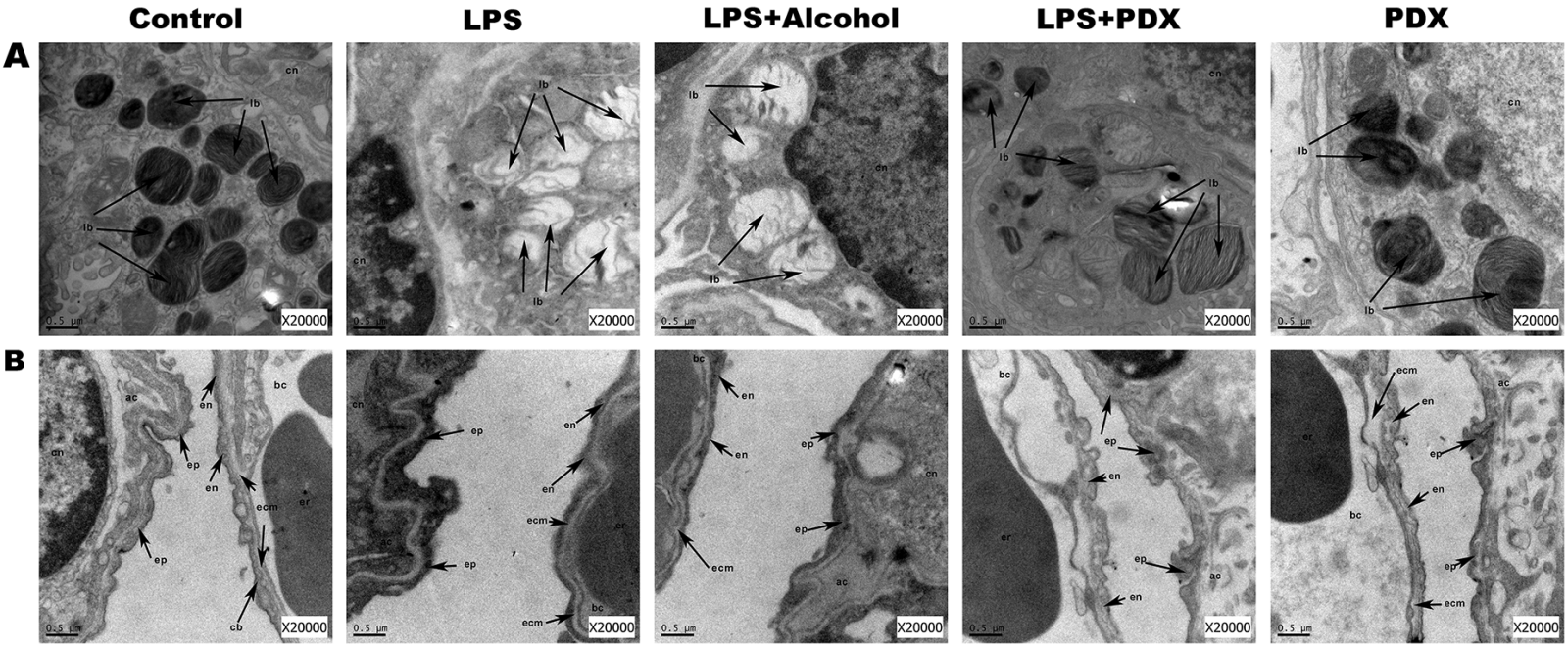

Control
LPS
LPS+Alcohol
LPS+PDX
PDX
A
B
C
cn
lb
ac
en
bc
ep
ecm
er
cb
0.5 μm
X20000
Alveolar Fluid Clearance (%/1 hour)
25
20
15
10
5
0
**
**
††††
††††
Control
LPS
LPS+Alcohol
LPS+PDX
PDX

Figure 3

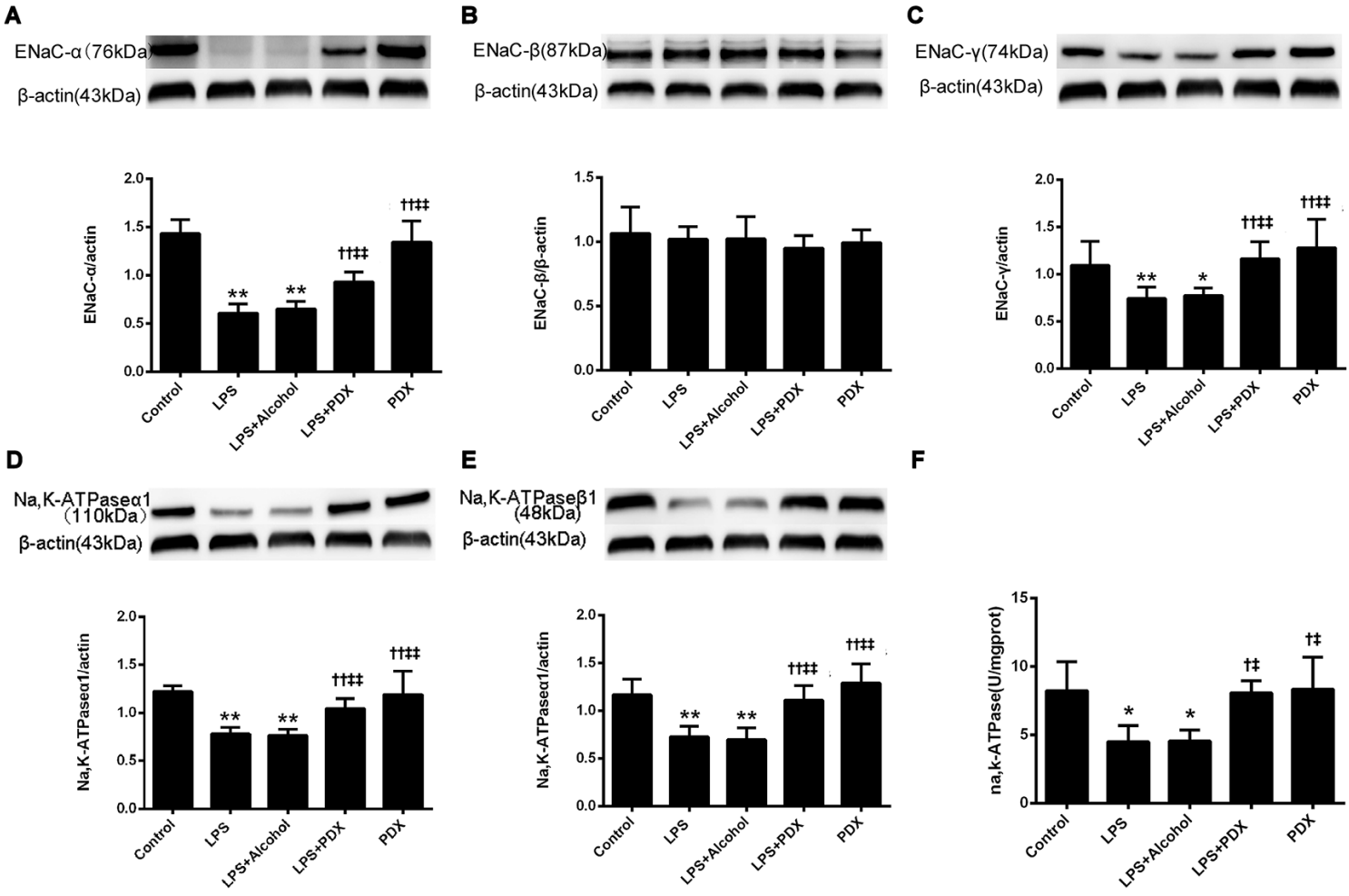
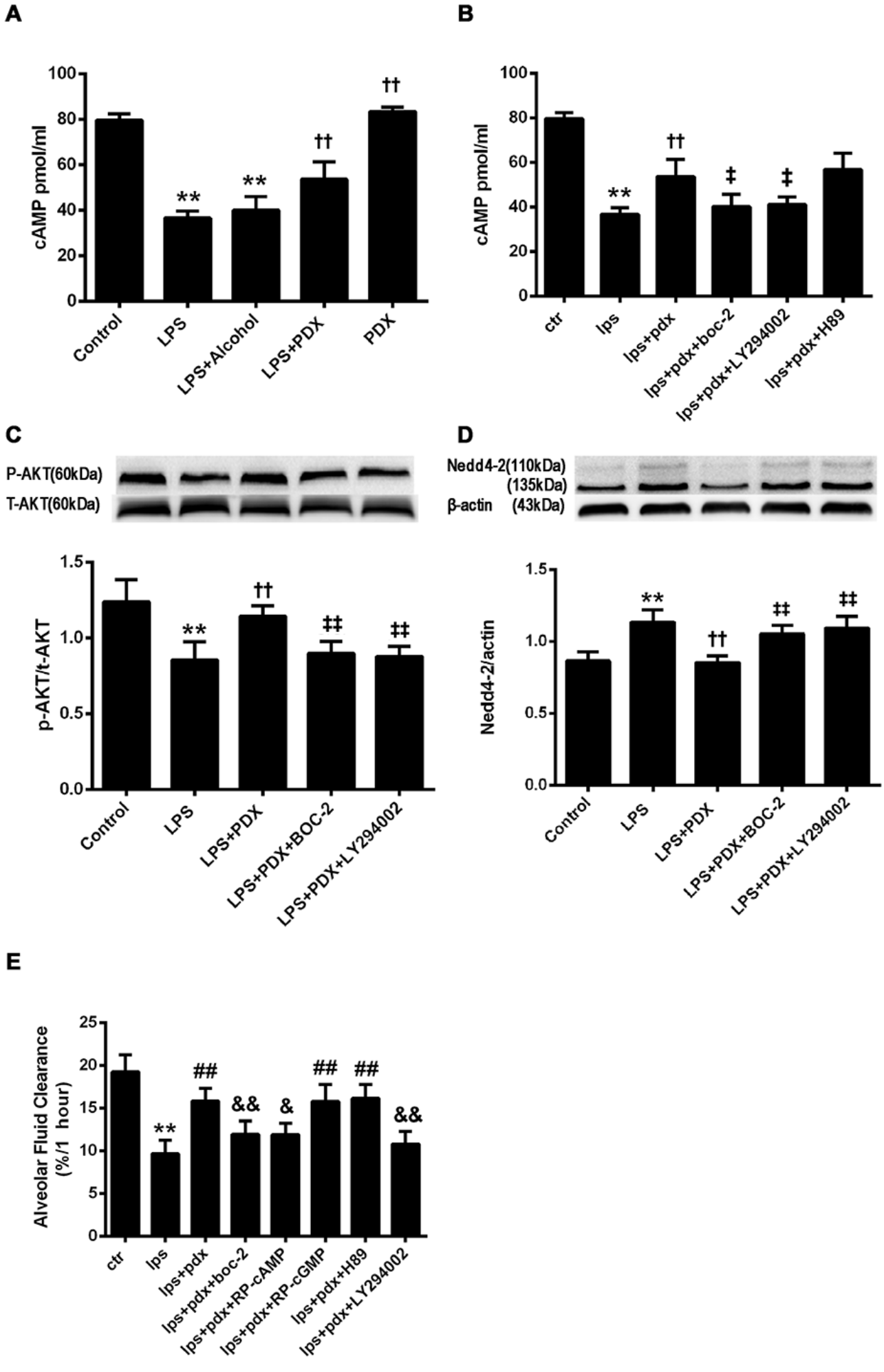

A
ENaC-α（76kDa）
β-actin(43kDa)
ENaC-α/actin
B
ENaC-β(87kDa)
β-actin(43kDa)
ENaC-β/β-actin
C
ENaC-γ(74kDa)
β-actin(43kDa)
ENaC-γ/actin
D
Na,K-ATPaseα1
（110kDa）
β-actin(43kDa)
Na,K-ATPaseα1/actin
E
Na,K-ATPaseβ1
(48kDa)
β-actin(43kDa)
Na,K-ATPaseα1/actin
F
na,k-ATPase(U/mgprot)
Control
LPS
LPS+Alcohol
LPS+PDX
PDX

Figure 4


A
cAMP pmol/ml
Control
LPS
LPS+Alcohol
LPS+PDX
PDX
B
cAMP pmol/ml
ctr
lps
lps+pdx
lps+pdx+boc-2
lps+pdx+LY294002
lps+pdx+H89
C
P-AKT(60kDa)
T-AKT(60kDa)
p-AKT/t-AKT
Control
LPS
LPS+PDX
LPS+PDX+BOC-2
LPS+PDX+LY294002
D
Nedd4-2(110kDa)
(135kDa)
β-actin (43kDa)
Nedd4-2/actin
Control
LPS
LPS+PDX
LPS+PDX+BOC-2
LPS+PDX+LY294002
E
Alveolar Fluid Clearance (%/1 hour)
ctr
lps
lps+pdx
lps+pdx+boc-2
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lps+pdx+RP-cGMP
lps+pdx+H89
lps+pdx+LY294002

Figure 5

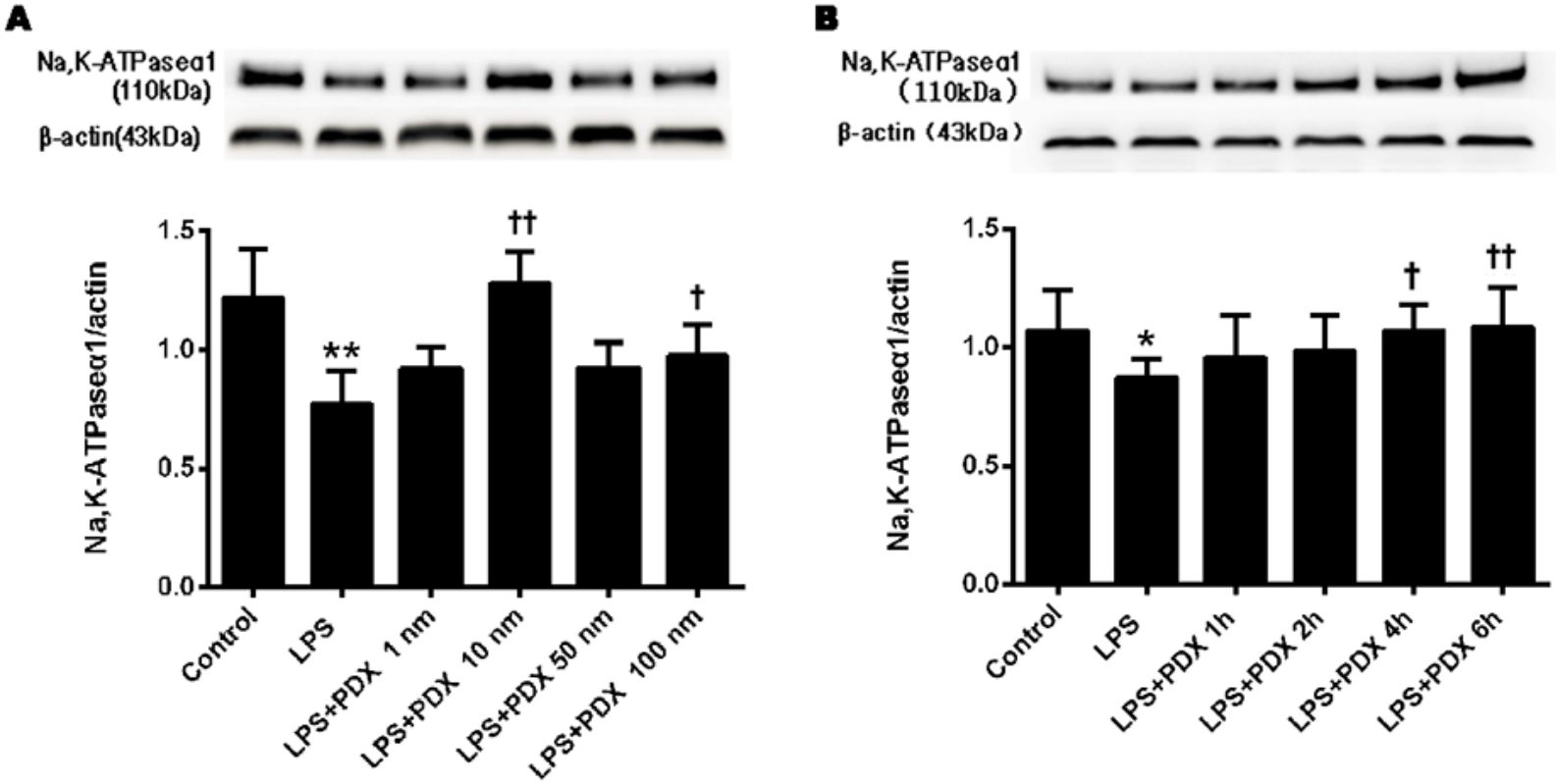

A
Na,K-ATPaseα1
(110kDa)
β-actin(43kDa)
Na,K-ATPaseα1/actin
1.5
1.0
0.5
0.0
**
††
†
Control
LPS
LPS+PDX 1 nm
LPS+PDX 10 nm
LPS+PDX 50 nm
LPS+PDX 100 nm
B
Na,K-ATPaseα1
(110kDa)
β-actin (43kDa)
Na,K-ATPaseα1/actin
1.5
1.0
0.5
0.0
*
†
††
Control
LPS
LPS+PDX 1h
LPS+PDX 2h
LPS+PDX 4h
LPS+PDX 6h

Figure 6

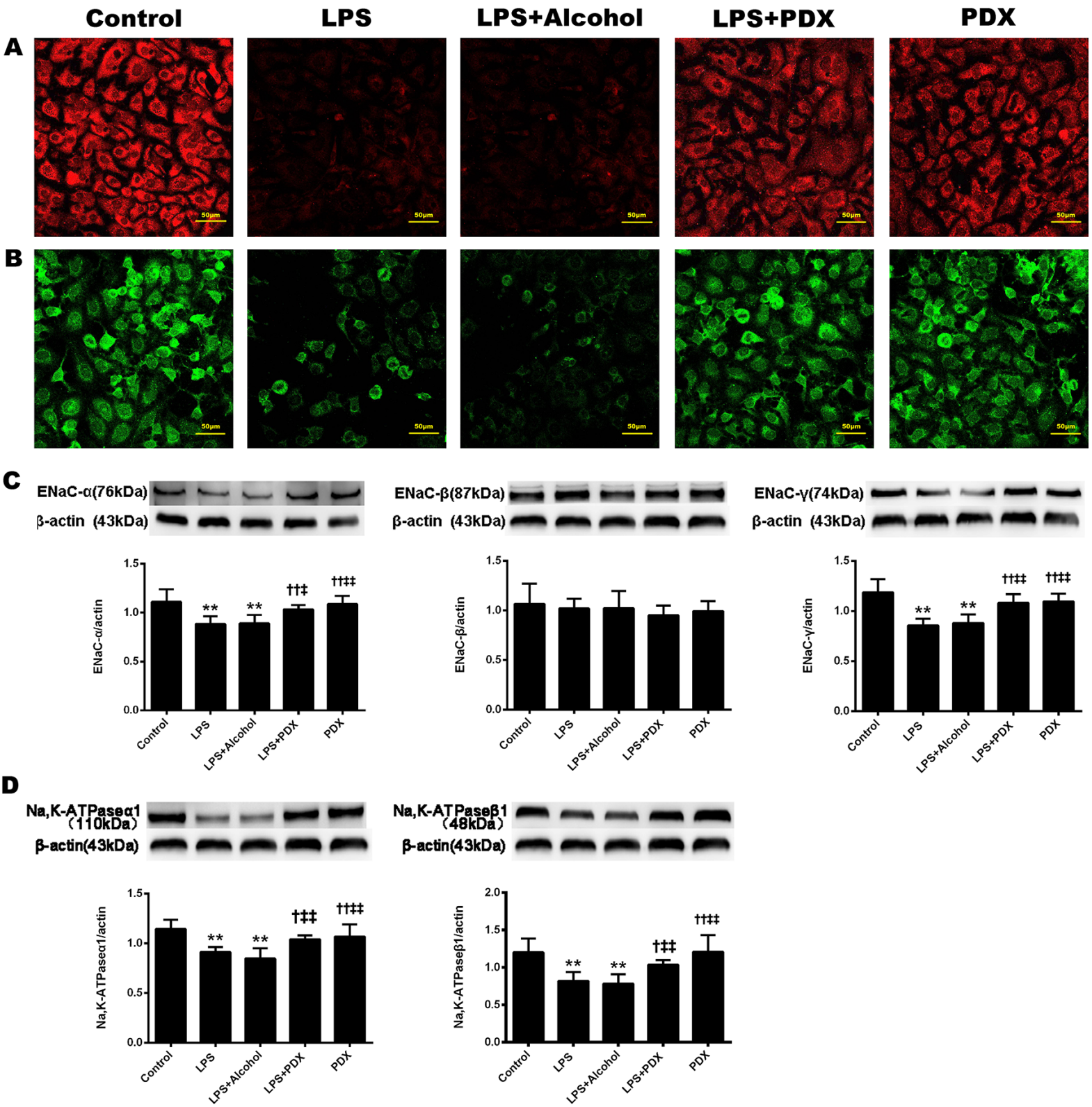
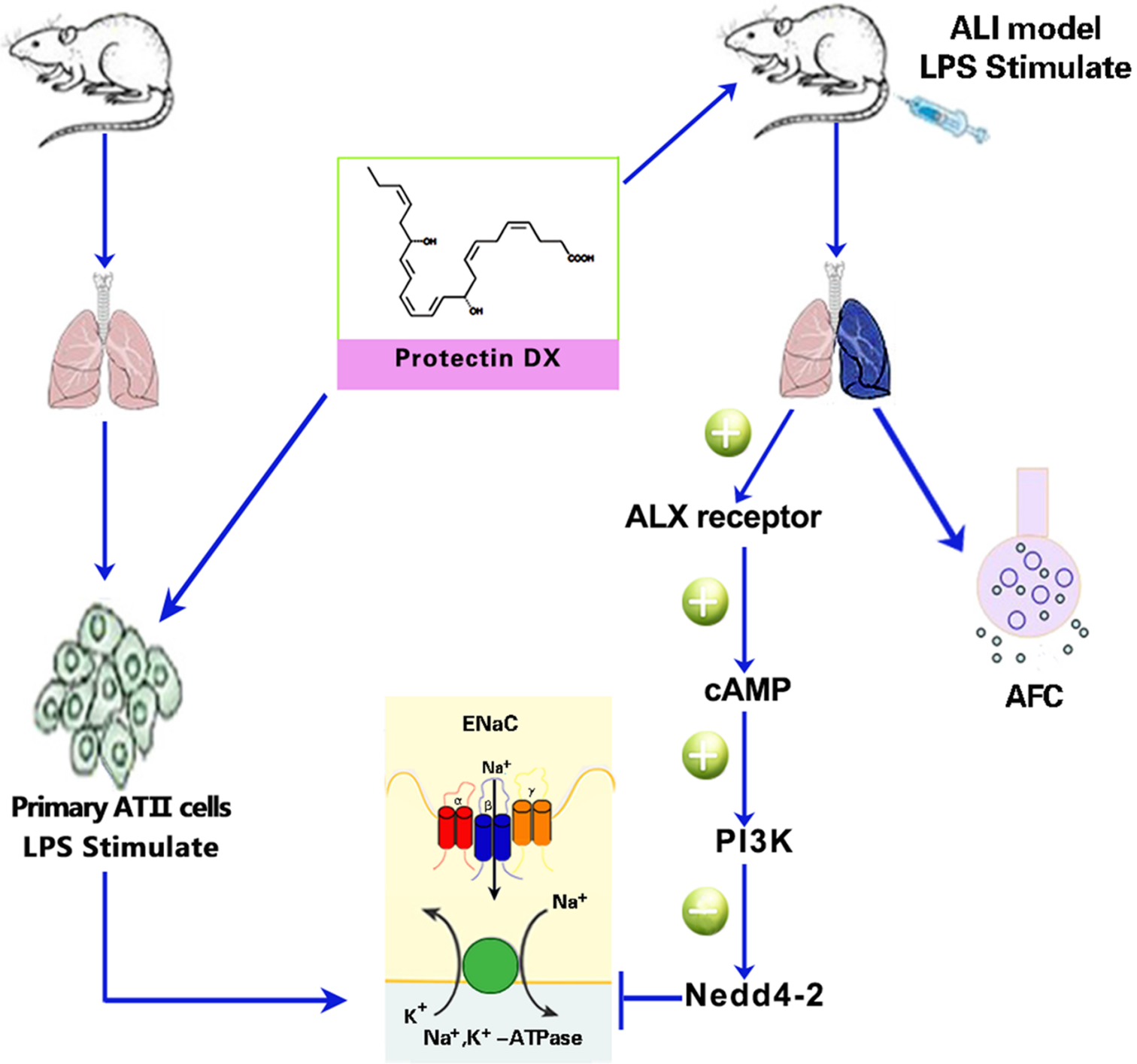
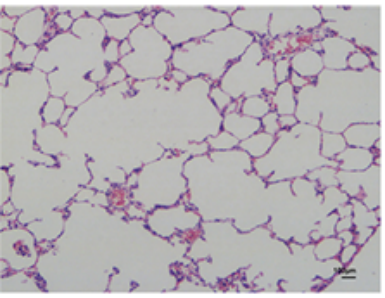

Control
LPS
LPS+Alcohol
LPS+PDX
PDX
A
B
50μm
C
ENaC-α(76kDa)
β-actin (43kDa)
ENaC-β(87kDa)
β-actin (43kDa)
ENaC-γ(74kDa)
β-actin (43kDa)
ENaC-α/actin
ENaC-β/actin
ENaC-γ/actin
1.5
1.0
0.5
0.0
**
†††
††††
Control
LPS
LPS+Alcohol
LPS+PDX
PDX
D
Na,K-ATPaseα1 (110kDa)
β-actin(43kDa)
Na,K-ATPaseβ1 (48kDa)
β-actin(43kDa)
Na,K-ATPaseα1/actin
Na,K-ATPaseβ1/actin
2.0
1.5
1.0
0.5
0.0
**
†††
††††
Control
LPS
LPS+Alcohol
LPS+PDX
PDX

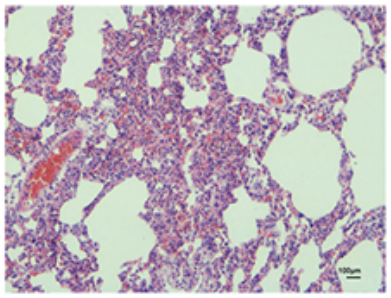
Figure 7


ALI model
LPS Stimulate
Protectin DX
ALX receptor
cAMP
PI3K
Nedd4-2
AFC
Primary ATII cells
LPS Stimulate
ENaC
Na+
Na+
K+
Na+,K+ –ATPase

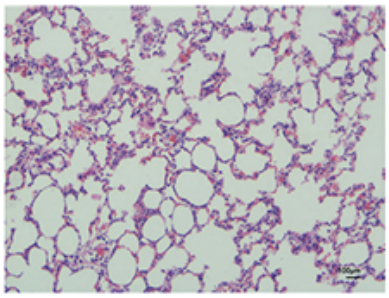
Supplemental Figure



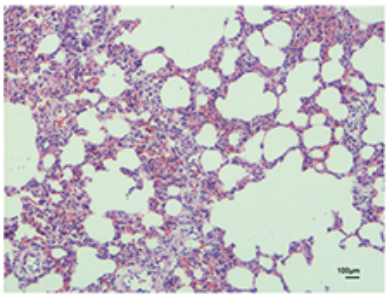
Control



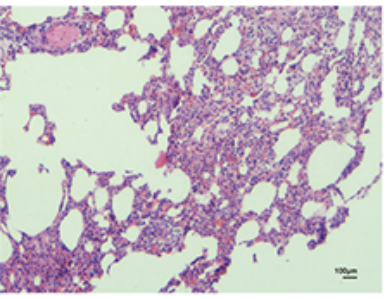
LPS



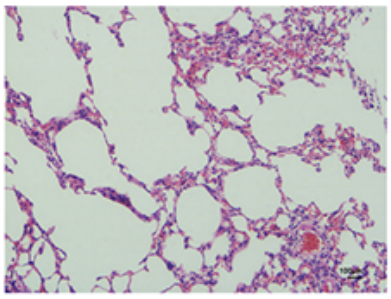
LPS+PDX



LPS+PDX+BOC-2



LPS+PDX+RP-cAMP



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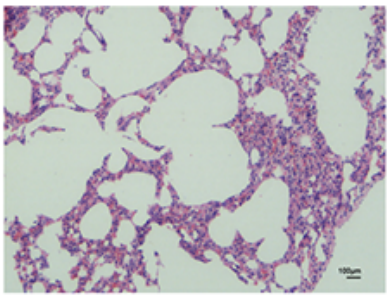
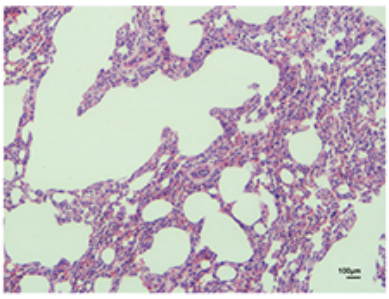


LPS+PDX+H89



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